

## Intracellular Pteroylpolyglutamate Hydrolase from Human Jejunal Mucosa

### ISOLATION AND CHARACTERIZATION\*

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Human jejunal intracellular pteroylpolyglutamate hydrolase was purified 30-fold from intestinal mucosa. The apparent molecular weight of the enzyme was 75,000 by Sephadex G-200 gel filtration, and the isoelectric point was at pH 8.0. The enzyme was maximally active at pH 4.5 and was unstable at increasing temperatures. Intracellular pteroylpolyglutamate hydrolase cleaved both terminal and internal  $\gamma$ -glutamate linkages. In contrast, brush-border pteroylpolyglutamate hydrolase catalyzed the hydrolysis of only terminal  $\gamma$ -glutamate linkages. The intracellular enzyme showed greatest affinity for the complete folic acid molecule with longer glutamate chains. Subcellular fractionation studies showed the intracellular enzyme was localized in lysosomes. These data show that the properties of human jejunal intracellular pteroylpolyglutamate hydrolase are distinct from those of the brush-border enzyme but are similar to the properties of intracellular pteroylpolyglutamate hydrolase described in other tissues.

polyglutamate hydrolase may play a role in cellular folate metabolism that is unrelated to the digestion of dietary folates.

### EXPERIMENTAL PROCEDURES<sup>1</sup>

#### RESULTS

**Physical Properties**—Intracellular pteroylpolyglutamate hydrolase was purified 30-fold (Table I). The apparent molecular weight was estimated by gel filtration to be 75,000. The isoelectric point was at pH 8.0. Maximal activity of the enzyme occurred at pH 4.5 (Fig. 1A) and at 65 °C (Fig. 1B). The enzyme was unstable at 37 °C in pH 4.5 assay buffer alone (Fig. 1C). However, the linearity of the product versus time curve for up to 45 min (Fig. 1D) indicated a protective effect of the substrate at 37 °C and ensured the validity of the enzyme assays. The activity of intracellular pteroylpolyglutamate hydrolase was unaffected by dialysis against 1 mM EDTA, and the addition of 100  $\mu$ M zinc acetate to the reaction mixture resulted in 15% inhibition.

**Affinity for Substrate**—The  $K_m$  for PteGlu,<sup>2</sup> determined from a Lineweaver Burk plot, was 1.2  $\mu$ M. Fig. 2 shows reciprocal plots of PteGlu hydrolysis in the presence of varied concentrations of PteGlu. This compound was a competitive inhibitor of the reaction, with a  $K_i$  of 0.08  $\mu$ M obtained from a plot of the  $x$  intercepts. PteGlu, also showed similar inhibition characteristics but had lower affinity for the enzyme, with a  $K_i$  of 1.2  $\mu$ M (data not shown). The effects of various PteGlu moieties on the activity of the enzyme is shown in Table II. Complete inhibition of PteGlu hydrolysis was observed with PteGlu and PteGlu<sub>2</sub> at 0.1 mM. Both PteGlu and H<sub>2</sub>PteGlu at 0.1 mM caused a 15% inhibition and at 1.0 mM, a 60% inhibition. At 1 mM, putine and  $\gamma$ -di-glutamate showed 50 and 30% inhibition, respectively. There was a slight inhibition by 1 mM *p*-aminobenzoyle-glutamic acid or no inhibition by 1 mM glutamic acid,  $\alpha$ -di-glutamic acid, or  $\alpha$ -tri-glutamic acid (Table II).

Pteroylpolyglutamate hydrolases catalyze the hydrolysis of pteroylpolyglutamates to derivatives with shorter glutamate chains. These enzymes have been described in the intestinal mucosa of several different species. Only the intracellular form of pteroylpolyglutamate hydrolase has been found in the intestinal mucosa of most animals, whereas two forms of the enzyme have been identified in human and pig intestinal mucosa. The first is associated with the brush-border membrane, and the second is soluble and in the intracellular fraction (1). We recently described the purification and properties of human brush-border pteroylpolyglutamate hydrolase and showed that this enzyme is involved in the digestion of pteroylpolyglutamate, the predominant form of dietary folate (2). Relatively little is known, however, about the properties of human intracellular pteroylpolyglutamate hydrolase and its possible role in folate metabolism. Our present objectives were to isolate and characterize intracellular pteroylpolyglutamate hydrolase from human intestinal mucosa and to compare its properties with those of the human intestinal brush-border enzyme. These data show distinct properties for each hydrolase and suggest that intracellular pteroyl-

\* Portions of this paper (including "Experimental Procedures" and Figs. 1-5) are presented in manuscript at the end of this paper. Manuscript is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86-M-0836, cite the authors, and include a check or money order for \$5.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

<sup>1</sup> The abbreviations used are: PteGlu, pteroyltri-glutamic acid; PteGlu<sub>2</sub>, pteroylbis-glutamic acid; PteGlu<sub>3</sub>, pteroyltri-glutamic acid; H<sub>2</sub>PteGlu, tetrahydropteroyltri-glutamic acid; PteGlu<sub>2</sub>OH, pteroyldi-glutamic acid; PteGlu<sub>2</sub>OH<sub>2</sub>, pteroyldi-glutamic acid; PteGlu<sub>2</sub>OH<sub>2</sub>OH, pteroyldi-glutamic acid; PteGlu<sub>2</sub>OH<sub>2</sub>OH<sub>2</sub>, pteroyldi-glutamic acid; BPLC, high pressure liquid chromatography.

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TABLE I

Procedure	Partial purification of intracellular pteroylpolylutamate hydrolase				Homogeneity	Purification (times)
	Total protein	Protein	Total activity	Specific activity		
	$\mu\text{g}$	$\mu\text{g}/\text{mg}$	milliunits	milliunits/ $\mu\text{g}$	%	times
10% homogenate*	4850	17.5	752	0.15	100	1
30,000 $\times$ g supernatant	1845	6.6	568	0.7	74	2
pH precipitation	940	4	486	0.12	64	3
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> precipitation	395	14.0	221	0.24	30	4
Isoelectric focusing	24	1.46	106	4.14	14	27.6

\* Homogenate was made from 20 g of tissue.

TABLE II

Effect of different mixtures of PteGlu<sub>2</sub> on PteGlu<sub>2</sub> hydrolase

Compound	% of control activity	
	0.1 mM	1 mM
PteGlu	82	34
H <sub>2</sub> PteGlu	82	40
PteGlu <sub>2</sub>	0	0
PteGlu <sub>3</sub>	0	0
p-Aminobenzoylglutamic acid	92	6
Pteridine	96	41
Glutamic acid	92	97
$\gamma$ -Glutamylglutamic acid	100	70
$\alpha$ -Glutamylglutamic acid	100	100
$\alpha$ -Glutamylglutaminylglutamic acid	100	100

**Mechanism of Hydrolysis.**—As shown in Fig. 3, the labeled products resulting from hydrolysis of PteGlu<sub>2</sub>[<sup>14</sup>C]Glu were equal amounts of [<sup>14</sup>C]glutamic acid and  $\gamma$ -glutamylglutamic acid. Time-course incubations of the enzyme with PteGlu<sub>2</sub> showed the rapid appearance of folic acid (PteGlu<sub>2</sub>) with minimal accumulation of intermediate products (Fig. 4). Incubation of the enzyme with PteGlu<sub>2</sub>[<sup>14</sup>C]GluLeu<sub>2</sub> resulted in a release of radioactivity that corresponded to 5% of the hydrolysis rate when using PteGlu<sub>2</sub> as substrate.

**Subcellular Location.**—Using fresh tissue, intracellular pteroylpolylutamate hydrolase was localized in the fractions enriched with mitochondria and lysosomes (Fig. 5A). Freezing and thawing of the tissue resulted in a similar redistribution of the lysosomal marker enzyme and intracellular pteroylpolylutamate hydrolase (Fig. 5B). More than 80% of both the lysosomal marker *N*-acetylglucosaminidase and pteroylpolylutamate hydrolase appeared in the soluble fraction. Other marker enzymes showed no change when compared to fresh tissue.

## DISCUSSION

The absorption of dietary folate is attributed in part to the activity of specific pteroylpolylutamate hydrolases located in the intestinal mucosa. To understand the mechanisms involved in absorption of dietary folate, we have focused our studies on two pteroylpolylutamate hydrolases in human intestinal mucosa. Recently, we reported on the purification and properties of the brush-border enzyme (2). In the present study, we have examined the properties of the intracellular pteroylpolylutamate hydrolase to understand the possible relationship of the two enzymes in folate digestion and metabolism.

As shown in Table I, a 30-fold purification of intracellular pteroylpolylutamate hydrolase was achieved. The enzyme has an apparent molecular weight of 75,000, optimal activity at pH 4.5, a  $pI$  of 8.0, and instability at increasing temperature. The inhibition of PteGlu<sub>2</sub> hydrolysis by PteGlu<sub>2</sub> ( $K_i = 1.7 \mu\text{M}$ ) and PteGlu<sub>3</sub> ( $K_i = 0.09 \mu\text{M}$ ) showed competitive inhibition patterns with Lineweaver-Burk plots, indicative of pre-existing affinity for longer chain pteroylpolylutamates. Inhibition of PteGlu<sub>2</sub> hydrolysis by PteGlu<sub>2</sub>, and to a lesser extent by other

TABLE III

Comparison of intracellular pteroylpolylutamate hydrolase and brush-border pteroylpolylutamate hydrolase

Property	Intracellular	Brush border
Apparent M.	75,000	700,000
pH optimum	4.5	6.5
$K_m$ for PteGlu <sub>2</sub> ( $\mu\text{M}$ )	1.7	7.2
Reducing agent requirement	Yes	No
Temperature stability	No	Yes
Metal requirement	No	Yes (Zn <sup>2+</sup> , Co <sup>2+</sup> )
$K_i$ for PteGlu <sub>2</sub> ( $\mu\text{M}$ )	1.7	0.6
$K_i$ for PteGlu <sub>3</sub> ( $\mu\text{M}$ )	0.09	0.6
Mechanism of hydrolysis	Cleaves both terminal and internal linkages	Exopeptidase
Final product	PteGlu	PteGlu
Localization	Lysosomes	Brush border

folate derivatives, and the lack of inhibition by  $\alpha$ -glutamates or other moieties suggest that the enzyme requires both the complete folic acid moiety and  $\gamma$ -glutamate linkage for activity. The enzyme is capable of cleaving both terminal and internal  $\gamma$ -peptide bonds since incubation of intracellular pteroylpolylutamate hydrolase with PteGlu<sub>2</sub>[<sup>14</sup>C]Glu resulted in the release of both [<sup>14</sup>C]labeled glutamic acid and [<sup>14</sup>C]labeled dipeptide. The release of radioactivity when the enzyme was incubated with PteGlu<sub>2</sub>[<sup>14</sup>C]GluLeu<sub>2</sub> and the minimal accumulation of the intermediate product with PteGlu<sub>2</sub> incubation support this conclusion. Subcellular fractionation studies using differential centrifugation demonstrated that the intracellular pteroylpolylutamate hydrolase is located in the lysosomes.

Comparisons of the properties of human intracellular and brush-border pteroylpolylutamate hydrolase indicate that they are distinct enzymes (Table III). The differences between these two enzymes include molecular weight, optimum pH, temperature stability, and requirement for metal ions and a reducing agent. Both enzymes showed similar  $K_m$  values for PteGlu<sub>2</sub> and greatest affinity when both the folic acid moiety and the  $\gamma$ -glutamate bond were present. However, intracellular pteroylpolylutamate hydrolase had greater affinity for folates with longer glutamate chains, whereas the brush-border enzyme had no preference for the number of glutamate residues. Whereas intracellular pteroylpolylutamate hydrolase is capable of cleaving both internal and terminal  $\gamma$ -glutamate linkages, the brush-border enzyme is an exopeptidase (2).

Comparisons of human intestinal intracellular pteroylpolylutamate hydrolase with pteroylpolylutamate hydrolases from other mammalian tissues reveal similarities and differences. Similar properties of pteroylpolylutamate hydrolases have been described in human liver (16), bovine liver (17), rat liver (18), hog kidney (19), guinea pig intestine (20), and rat intestine (21). In each site, the enzyme had an acidic pH optimum and was demonstrated to be lysosomal in human

liver, rat liver, and guinea pig intestine. The ability to cleave internal  $\gamma$ -glutamate bonds was observed in studies of pteroylpolyglutamate hydrolase isolated from bovine liver and rat intestine, whereas exceptional activity was observed in human liver and hog kidney. Affinity toward longer glutamate chains was observed in both bovine liver and rat intestine. Furthermore, sensitivity to sulfhydryl agents and the protective effect of reducing agents were properties of pteroylpolyglutamate hydrolase from human liver, bovine liver, and hog kidney, which suggests involvement of SH groups in activity.

The role of intracellular pteroylpolyglutamate hydrolase in the human intestinal mucosa is obscure. A possible involvement of the intracellular pteroylpolyglutamate hydrolase in the absorption of dietary folate is not excluded but would require transport of all or part of the pteroylpolyglutamates into the cell prior to hydrolysis. Intracellular pteroylpolyglutamate hydrolase may function in regulating the levels of pteroylpolyglutamates within the erythrocyte since others have demonstrated the capability for synthesis of these forms of the vitamin by intestinal mucosa (22). The similarities between human intestinal intracellular pteroylpolyglutamate hydrolase and the intracellular enzyme from other mammalian tissues imply that these enzymes have similar roles in cellular folate metabolism. Furthermore, pteroylpolyglutamate is not only the preferred coenzyme for many folate-dependent enzymes in single carbon transfer reactions but also has been found to be an effective inhibitor of a number of enzymes, including thymidylate synthetase and methylenetetrahydrofolate reductase (23, 24). Others observed increased glutamylolysis of folate in hepatoma cells in the presence of insulin or dexamethasone (25). These observations suggest that a fairly complex regulation of pteroylpolyglutamate levels exists in the cell and implies that jejunal mucosal intracellular pteroylpolyglutamate hydrolase may play a significant physiological role in cellular metabolism.

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